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(54) Title: A PEPTIDE PREPARATION, A PROCESS FOR PRODUCING IT AND USE OF THE PEPTIDE PREPARATION

(57) Abstract

A peptide preparation from hydrolysis of whey containing peptides with a molecular weight of up to 6.000 Dalton. Such a preparation is hypoallergenic and therefore useful in food products and stimulants, such as mother's milk substitutes, edible ice, protein beverages and other products, usually containing milk or milk protein, in particular for allergics or humans with lactose malabsorption. The preparation is produced by a combination of enzymatic hydrolysis and ultrafiltration.

A peptide preparation, a process for producing it and use of the peptide preparation

The present invention concerns a peptide preparation from hydrolysis of whey, a process for producing it by ultrafiltration and hydrolysis of whey as well as use of the peptide preparation as a substitute for milk or milk protein.

In the population there are increasing problems with allergy, i.a. milk allergy, just as there are some people who cannot absorb lactose. The problems are particularly great in case of infants, since milk and milk products constitute a significant proportion of their nutrition if they are not breast-fed for some reason.

It has been attempted to produce mother's milk substitutes from e.g. whey by a combination of enzymatic hydrolysis, heat treatment and ultrafiltration, cf. the US Patent Specification No. 4,293,571. The peptides obtained hereby have a size of 1,000 - 10,000 Dalton. However, it is well-known that peptides of 5,000 - 10,000 Dalton are often allergenic, see e.g. Immunochemistry, Pergamon Press 1967, 4, p. 1-10.

The process described in the mentioned US patent specification comprises denaturation of the proteins, which is performed by a heat treatment, e.g. at 100 - 140°C for from 10 seconds to 4 minutes or at 75 - 100°C for 2 - 60 minutes. Such a strong denaturation involves a great risk of destruction of sulfur-containing amino acids, formation of Maillard reaction products which are often allergenic (hapten effect), just as the taste of the product is impaired. See e.g. Otani, H. and Tokita, F., Jap. J. Zootechn. Sci., 1982, 53, p. 344, Otani

et al., Jap. J. Zootechn. Sci., 1985, 56, p. 987 and Matsuda, T. et al., J. Food Sci., 1985, 50, p. 618.

It has now been found that if a whey-based peptide preparation comprising peptides with a molecular weight of up to 6,000 Dalton is provided, it may be incorporated as a component in food products and stimulants for allergics. The preparation is useful in mother's milk substitutes for infants as well as in food products and stimulants for persons liable to develop allergy, persons with lactose malabsorption, as a liquid diet for patients having gone through gastric/intestinal surgery, as well as a protein source for athletes, in particular weight lifters, throwers and bodybuilders. It is useful in stimulants such as ice and protein beverages.

Thus, the preparation of the invention is characterized by comprising peptides with a molecular weight of up to 6,000 Dalton and by being free of allergenic substances, such as Maillard reaction products and lactose.

A particularly preferred peptide preparation is characterized by substantially consisting of peptides with a molecular weight of 2,000 - 6,000 Dalton, in particular 2,000 - 2,500 Dalton, since small molecules, such as free amino acids and salts tend to cause diarrhoea because of their osmotic effect, which is undesirable of course.

The invention also concerns a process for producing the present peptide, which is characterized by

a) diafiltrating essentially casein-free whey with water on an about 20,000 Dalton membrane, if desired after a preceding concentration of the whey,

b) enzymatically hydrolyzing the whey protein retentate

from a) in one or more steps, each hydrolysis step being terminated with ultrafiltration through an about 6,000 Dalton membrane to harvest the resulting peptides in the permeate.

If the whey used as a starting material is not essentially casein-free, it must, prior to performing the process of the invention, be subjected to coarse filtration, centrifugation or other treatment for removal of casein residues which may perhaps be present as complexes. The whey is expediently purified on a 200,000 Dalton membrane, i.e. a membrane with a cut-off value of 200,000 Dalton, or by a pH adjustment to about 4.6 and addition of a precipitant, preferably calcium chloride. Any membrane with the desired cut-off value may be used here and throughout the description and claims. Usually, commercially available membranes are preferred, produced from high-resistant synthetic polymers which are also useful for clean-in place, CIP cleaning, i.e. cleaning on site, with e.g. acid and lye. Such membranes are available e.g. from DDS, De Danske Sukkerfabriker, Rhône-Poulenc and Romicon. The membranes used in the examples are from DDS.

The EP Patent Application No. 65663 discloses a process for producing a protein hydrolysate useful as a food for patients on an enteric diet. The process comprises hydrolysis of a whey protein suspension with a lactose content of no more than 1.0% by weight with a protease from Aspergillus niger, preferably after a basic pre-hydrolysis at 90 - 95°C. If desired, a bacterial protease from Bacillus licheniformis may also be used. The suspension is heat treated after the hydrolysis, typically to 90°C to inactivate the enzyme or enzymes.

Such a product will have a considerable proportion of substances with a molecular weight below 2,000 and will

therefore tend to cause diarrhoea. Further, there will be a non-negligible content of proteins with a molecular weight above 6,000 which are allergenic. In particular, the heat treatment for inactivating the enzymes will cause Maillard reaction products with a hapten effect.

Conclusively, the described hydrolysate will therefore not be suitable for use as a milk substitute for milk allergics and infants, just as it does not appear to be useful for humans who cannot tolerate lactose.

Further, the EP Patent Specification No. 33694 discloses a process for producing a stable hydrolysate of animal proteins, wherein the protein hydrolysate is mixed with a liquid milk product, e.g. a retentate from ultrafiltration of milk in amounts of at least 50% based on the stabilized protein hydrolysate.

The stabilized hydrolysates are intended for dietetic nutrition of sick people and malnutritioned and undernourished people, but cannot readily be tolerated by milk allergics and humans with lactose malabsorption.

By omitting a denaturing heat treatment as described in the US Patent Specification No. 4,293,571 and the EP Patent Application 65663 and otherwise proceeding according to the process of the invention, a product will be obtained which is hypoallergenic and lactose-free.

The EP Patent Application No. 22019 admittedly describes a total enzymatic whey protein hydrolysate essentially free of residual proteins, defined by having no content of fractions which can be precipitated with 12% trichloroacetic acid. At least 50% of the peptides contains 2-5 amino acids, while the content of free amino acids is lower than 15%. Preferably, 70 - 90% of the peptides

has a chain length below 10.

The products are reported to be useful as food products, in particular post-surgery treatments with a view to re-establishing the metabolism as they may be absorbed directly through the intestinal wall and stimulate the enzyme production. Nothing is said about allergy problems, lactose malabsorption, nutrition of infants, i.e. the phenomena on which the proposed uses of the peptide preparation of the invention are based in particular, and the stated limitation criteria are not sufficient to ensure a hypoallergenic and lactose-free product.

The recovery of the hydrolysate takes place by a method having certain points of similarity to the process of the invention, since it involves enzymatic hydrolysis of an optionally ultrafiltrated and/or diafiltrated whey protein to the desired low molecular sizes, and collection of the desired hydrolysate by ultrafiltration without intermediate enzyme inactivation. However, the enzyme used is obligatory an enzyme capable of re-establishing the human protein metabolism, preferably pancreatin which is a mixture of particularly trypsin and chymotrypsin.

However, as mentioned, the patent application is silent on the uses of the molecular sizes critical to the usefulness of the preparation of the invention, just as it does not describe the combination of several enzymes by the hydrolysis.

According to a preferred embodiment of the process of the invention, the starting material used is whey from acid-precipitated casein. This results in a better taste than with e.g. whey from cheese production which, because of a certain content of casein, including kappa casein,

tends to give a bitter taste which may be difficult or impossible to remove.

The hydrolysis may be performed with various proteases, all of which - depending upon the quality and production conditions of the starting materials - can give a satisfactory peptide preparation. The hydrolysis may be followed continuously by the pH-stat method, and the degree of hydrolysis is calculated on the basis of the consumption of NaOH in the usual manner. The degree of hydrolysis is stated as the percentage proportion of cleaved peptide bonds in relation to the total number of peptide bonds, cf. J. Adler-Nisson: J. Chem. Technol. Biotechn. 32.138, 1982. The proteases are not inactivated prior to ultrafiltration. Virtually all proteases acceptable in the food industry may be used. Examples of particularly useful enzymes include enzymes from Aspergillus oryzae, such as "Rhozyme 41 PC", and from Bacillus licheniformis, such as "Alcalase® 0.6 L" and "Corolase PP" (pancreas extract). "Rhozyme 41 PC" contains leucine aminopeptidases which hydrolyze bitter peptides and is thus useful for the treatment of whey containing small amounts of bitter substances. The hydrolysis is preferably performed in one or more steps with intermediate ultrafiltration(s). This results in a better yield and a better molecular weight distribution.

I. Processing of whey protein

As mentioned, the best starting material is whey from acid precipitated casein. It is also possible to use whey from cheese production. The latter whey contains varying amounts of casein. Only whey containing small amounts of casein can be used since hydrolysis of casein leads to bitter substances, which may be difficult or impossible to remove. As mentioned, the whey is freed

of any residues of casein, e.g. by course filtration (200,000 Dalton), and/or by acid addition, preferably to a pH of about 4.6, and addition of a precipitant, preferably $CaCl_2$, in particular in an amount of 5-40 g/100 l whey and subsequent removal of the casein e.g. by decanting. Then, the permeate containing whey proteins may be concentrated (20,000 Dalton) to reduce the amount of liquid to be treated. High flow rates are obtained at a pH of 2.0 - 2.5. The concentrate is diafiltrated with water to remove lactose, increase pH and concentrate the protein content to 0.5% - 20%. The production of lactose-free whey protein concentrate, WPC, may be effected continuously. The production proceeds most expediently either cooled to about 5°C or heated to about 50°C to prevent microbial growth. If the whey from acid-precipitated casein or acid and $CaCl_2$ treated whey only has an insignificant content of casein, coarse filtration of the starting material is superfluous.

II. Production of hydrolysates

Whey protein concentrate may be hydrolyzed with various proteases, all of which can give a satisfactory peptide preparation, depending upon the quality and product conditions of the starting material, as mentioned. The hydrolysis may be followed continuously and the degree of hydrolysis is calculated on the basis of the consumption of NaOH. The minimum degrees of hydrolysis to be obtained are stated below. The proteases are not inactivated prior to ultrafiltration.

IIa. Hydrolysis with two enzymes

First, 5% whey protein concentrate is hydrolyzed at 50°C and pH 7.5 with 1.0 g of "Rhozyme 41 PC" (Aspergillus oryzae - Genencore) per litre of substrate to 9% hydro-

lysis. Ultrafiltration is performed on a 6,000 Dalton membrane.

The yield of peptide in the UF permeate constitutes 30 - 40% of the initial protein amount.

Then the retentate from ultrafiltration of the "Rhozyme" hydrolysate is further hydrolyzed at 50°C and pH 7.5 with 0.5 ml of "Alcalase® 0.6 L" (Bacillus licheniformis - NOVO) per litre of substrate (start volume at the first hydrolysis). The hydrolysis is continued to 12%. The yield of peptide in the UF permeate is 40 - 45% of the initial protein amount. The total yield is 70 - 80%.

The sequential hydrolysis increases the overall yield. The intermediate ultrafiltration is used for harvesting peptides formed in the first hydrolysis, which would otherwise be hydrolyzed further to free amino acids with a resulting higher osmolarity of the product.

IIb Combined hydrolysis with two enzymes

Advantageously both enzymes are used in one step, since this leads to an improved yield and the intermediate harvesting is avoided.

IIc. Hydrolysis with "Corolase PP"

Whey protein concentrate may also be hydrolyzed with "Corolase PP" (pancreas extract - Rohm) at 50°C and pH 8.0 (5.0 g per kg of whey protein concentrate) to 10% hydrolysis. However, this requires casein-free whey protein concentrate to avoid formation of bitter peptides. A yield of 50 - 60% peptides in the UF permeate is obtained.

IId. Hydrolysis with "Alcalase 0.6 L"

5% whey protein concentrate may be hydrolyzed at 50°C and pH 7.5 with "Alcalase® 0.6 L" (1.0 ml per litre of substrate) to 18% hydrolysis. The yield of peptide in the UF permeate is about 80%. However, the method can only be used on essentially casein-free whey protein since casein leads to formation of bitter peptides.

III. Harvest of peptides

The hypoallergenic peptides are harvested from the hydrolysates by ultrafiltration and/or diafiltration, a combination being often preferred since fouling during filtration reduces the permeability of the filter.

The peptides produced by the process of the invention may be used in various food products and stimulants as mentioned. Thus, mother's milk substitutes may be produced by adjusting the protein content in the harvested UF permeate to 3.0%, adding salts, vitamins, saccharose, starch and fat, and then the mixture is emulsified, concentrated, sterilized and atomization-dried to provide a finished product to be diluted when used. It is also possible to produce a liquid concentrate or a product ready for use, in a manner known per se.

The invention will be explained more fully by means of the following examples:

EXAMPLE 1

Production of mother's milk substitutes on the basis of whey from acid-precipitated casein

50,000 litres of casein-free whey from acid-precipitated casein with a protein content of 0.5% are pre-treated

by coarse filtration on "GR30PP" membranes (200,000 Dalton). Then the whey proteins are concentrated and diafiltrated on "GR61PP" membranes (20,000 Dalton). The resulting retentate is free of lactose. The retentate is then fed to a tank equipped with a heat jacket to keep a constant temperature of 50°C. The pH of the whey protein concentrate is adjusted to 8.0 with the pH meter /titrator unit used in the hydrolysis to keep a constant pH of about 8.0. The consumption of NaOH is used as a measure of the degree of hydrolysis. The tank is moreover equipped with an efficient stirrer. When the whey protein concentrate has been equilibrated at 50°C and pH 8.0, 1.23 kg of "Corolase PP" are added, and the hydrolysis proceeds until about 40 litres of 4.0 N NaOH (10% hydrolysis) have been consumed. This takes about 4 hours. Then the hydrolysate is ultrafiltrated/diafiltrated on "GR81PP" membranes (6,000 Dalton) to provide a hypoallergenic permeate. The protein content in the permeate is measured continuously at 280 nm. The protein content in the permeate is 2.5 to 3.0%. 125 kg of hypoallergenic peptide are obtained, corresponding to 1,000 kg of complete mother's milk substitute.

EXAMPLE 2

Production of hypoallergenic peptide on the basis of whey from cheese production

19,200 litres of whey from cheese production with a protein content of about 0.65% are coarse filtrated as in example 1, or treated with an acid and $CaCl_2$, as stated before. The permeate, constituting whey cleaned of particulate casein, but containing kappa casein, is fed to another tank and concentrated/diafiltrated as in example 1. Then the retentate constitutes lactose-free whey protein concentrate. The whey protein concen-

trate is adjusted to pH 7.5 and 50°C, and 2.5 kg of "Rhozyme 41 PC" are added. The hydrolysis is continued until about 18 litres of 4.0 N NaOH (9% hydrolysis) have been consumed, which takes about 2 hours. The hydrolysate is ultrafiltrated/diafiltrated as stated in example 1. The yield of peptides in the permeate is about 35%. The retentate is adjusted to pH 7.5 and 50°C, and 1,25 litres of "Alcalase® 0.6 L" are added. The hydrolysis is continued until about 15 litres of 4.0 N NaOH (12% hydrolysis of the retentate) have been consumed, which takes about 2 hours. The "Alcalase 0.6 L" hydrolysate is filtrated/diafiltrated as stated in example 1. The yield of peptides in the UF permeate is about 35% of the whey protein concentrate amount. The protein content in the total UF permeate after hydrolysis with the two enzymes will be below 1.0%, and the UF permeate is therefore concentrated 4 - 5 times before it is suitable for use in the production of mother's milk substitutes. 90 kg of hypoallergenic peptide are obtained, corresponding to 760 kg of complete mother's milk substitute.

EXAMPLE 3

60,000 litres of whey from cheese production having a protein content of 0.85% is pre-treated by lowering of pH to 4.6 and precipitation with $CaCl_2$. Precipitated casein is removed by decanting, and the liquid having a protein content of 0.70% is diafiltrated so that the lactose content is reduced to max. 0.1%, and then the protein content is adjusted to 5%. This retentate is heat-treated at 70°C for 10 minutes to inactivate rennet, and then "Rhozyme" and "Alcalase® 2.4 L" are added simultaneously in amounts corresponding to E/S 2 and 1.36%, respectively. The hydrolysis is performed at a pH of 7.5 and at 55°C, and pH is kept at 7.5 with a pH-stat during the hydrolysis.

A degree of hydrolysis of about 17% is obtained after about 4 hours, and then the mixture is ultrafiltrated on a membrane with a cut-off value of 6,000.

About 4,500 litres of permeate having a protein content of about 3.5% is obtained. This is processed by addition of carbohydrates, vitamins, fat and minerals in accordance with the regulations in this field to provide a hypoallergenic lactose-free preparation, which may be further processed by sterilisation and/or spray-drying.

When using two tanks, the process may be performed continuously as hydrolysis may take place in one tank, while ultrafiltration/diafiltration of whey protein takes place in the other tank. The process described in example 1 is considerably less expensive with respect to consumption of enzymes and requirement of apparatus than the process described in example 2. On the other hand, the requirements made of the quality (and thus price) of the optionally concentrated whey used as the starting material are lower in example 2, just as the yield in example 2 is higher than in example 1. An even higher yield may be obtained by a process corresponding to example 1 by using a thorough hydrolysis with "Alcalase 0.6 L". However, this process is very exacting with respect to the whey, which, as mentioned, must be essentially free of casein. At present the process according to example 3 is preferred, due to its conveniency and the excellent quality of the product, which is both hypoallergenic, lactose-free and of attractive taste.

The products produced by the process of the invention show no allergenicity according to measurements, no antibody binding being observed in ELISA using the following antibodies:

I. Commercial rabbit antibodies, produced by the applicants, against casein or whey protein.

II. Sera from cow's milk allergic patients containing human antibodies against milk proteins.

Moreover, they cannot cause PCA reaction in mice passively sensibilized with rabbit or mouse antibody against milk proteins, they cannot cause positive reaction in in vitro Histamin-Release tests using patient sera as stated in the foregoing, and they do not cause allergy reactions in provocation tests on infants with clinically well-documented milk allergy. The peptides have an optimum content of essential amino acids and thus a high nutritional value, just as the product is lactose-free. A 1.5% aqueous solution of the peptides is tasteless and odourless. The peptides in an up to 10% aqueous solution are soluble at pH 4,5. At this pH the peptides tolerate boiling for 10 minutes. As mentioned, the peptides have a size below 6,000 Dalton, and most of them have a molecular weight of 2,000 - 2,500 Dalton. They have no or a very weak emulsifying capability, just as they are sparingly foaming with a poor foam stability. They are therefore useful as a substitute for milk, as mentioned in the foregoing.

PATENT CLAIMS

1. A peptide preparation from hydrolysis of whey, characterized by comprising peptides with a molecular weight of up to 6,000 Dalton, and optionally amino acids, and by being free of allergenic substances and lactose.

2. A peptide preparation according to claim 1, characterized by substantially consisting of peptides with a molecular weight of 2,000 - 6,000 Dalton, in particular 2,000 - 2,500 Dalton.

3. A process for producing a preparation according to claim 1 or 2 by ultrafiltration and hydrolysis of whey, characterized by

a) diafiltrating essentially casein-free whey with water on an about 20,000 Dalton membrane, if desired after a preceding concentration of the whey,

b) enzymatically hydrolyzing the whey protein retentate from a) in one or more steps, each hydrolysis step being terminated with ultrafiltration through an about 6,000 Dalton membrane to harvest the resulting peptides in the permeate.

4. A process according to claim 3, characterized by using whey from acid-precipitated casein.

5. A process according to claim 3 or 4, characterized by using whey made essentially casein-free by coarse filtration e.g. through a 200,000 Dalton membrane, or by addition of acic and precipitation with calcium chloride.

6. A process according to claim 3, 4 or 5, c h a r a c t e r i z e d by performing the hydrolysis in one or two steps with two different enzymes.

7. A process according to any of claims 3-6, c h a r a c t e r i z e d by using one or more proteases.

8. A process according to any of claims 3-7, c h a r a c t e r i z e d by performing the hydrolysis in one or two steps using "Rhozyme 41 PC" and "Alcalase ® 0.6 L".

9. Use of the peptide preparation according to claim 1 in a suitable concentration as a substitute for milk or milk protein in products usually containing milk.

10. Use according to claim 9 in products intended for humans with allergy or tendency to allergy and/or lactose malabsorption tendency.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	US, A, 4 107 334 (PFIZER INC.) 15 August 1978 See example 2.	1-10

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

II Fields Searched (cont).

US Cl 426:7, 41, 42

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ..... because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers ..... because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers ..... because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.